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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Yang, Yinong

Serial No.: 10/768,886 Art Unit: 1638

Filed: January 31, 2004 Examiner: Vinod Kumar

For: Mitogen-Activated Protein Kinase And Methods for Use to Enhance Biotic And Abiotic Stress Tolerance in Plants

Atty Docket No.: UAF-03-14

SUPPLEMENTAL DECLARATION OF YINONG YANG, PH.D. UNDER 37 C.F.R. §1.131

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

I, Yinong Yang certify the following:

1. I am the inventor of U.S. Patent Application No. 10/768,886.
2. The data filed with this declaration was generated from work performed in my laboratory by me or under my direct supervision at the University of Arkansas located in Fayetteville, Arkansas.
3. Prior to 2002, I completed my invention as described and claimed in the above referenced application as evidence below.
4. On or about May 2000, my laboratory isolated the gene fragment of OsMAPK5 (plasmid clone #2C12) (see attached Exhibit A, Lab Notebook I at pages 1-2).
5. On or about September 2000, my laboratory isolated the full length gene of OsMAPK5 (plasmid clone #M2) (see attached Exhibit A, Lab Notebook I at pages 3-5).

6. From approximately November 2000 to May 2001, RNA and protein analysis of OsMAPK5a indicating response to biotic and abiotic stresses were performed in my laboratory (see attached Exhibit A, Lab Notebook I at pages 6- 7).
7. On or about November 2000, rice transformation was initiated for over-expression (H series) and suppression (F series) of OsMAPK5.
8. On or about May 2001, my laboratory began to obtain transgenic rice lines (see attached Exhibit B, Lab Notebook II at page 1).
9. During approximately, June 2001 to May 2002, two generations for transgenic rice lines were analyzed for disease resistance and abiotic stress tolerance (see attached Exhibit B, Lab Notebook II at pages 2-4).
10. Prior to studies in my laboratory, no one in the field was aware that rice MAPK5 gene, its protein and enzyme activity were induced by drought, salt and low temperature and capable of rendering abiotic stress tolerance.

I certify that the foregoing statements made by me are true. I am aware that if any of the foregoing statements made by me is willfully false, I am subject to punishment.

Date: 9/25/2007

Yinong Yang Ph.D.

Department Plant Pathology

Subject Rice Defense gene Screening and Identification

Name Lizhong Xiong (NTI)

Address R. APC 215

National® Brand 99.10 — 2001.2

Computation Notebook

11 3/4" x 9 1/4", 4 x 4 Quad., 75 Sheets 43-648

0 73333 43648 8

I

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May 7 Rice seeds (Drew) planting
53 g → ~~60~~ 60 ~~plots~~ pots

May 4

Blots	probe
N5-1 N6-1 Minwoo's chemical induced (including CHX) seedlings	BTH10
N5-2 N6-2 Minwoo's blots suspension cell	JA60

May 8-9 cultures & Mini prep / sequencing
↓ all $A_{260/280} \geq 1.8$ but ≤ 1.95

1	2A10	0.32 μg/μl	11	2D10	0.29	21	2F11	0.34
2	2A12	0.34	12	2E2	0.31	22	2G1	0.32
3	2B1	0.28	13	2E3	0.30	23	2G5	0.37
4	2B7	0.36	14	2E4	0.5	24	2G6	0.38
5	2C1	0.30	15	2E7	0.41			
6	2C3	0.46	16	2E8	0.42			
7	2C4	0.38	17	2E11	0.37			
8	2C12	0.36	18	2F7	0.40			
9	2D2	0.46	19	2F8	0.29			
10	2D7	0.28	20	2F9	0.41			

Blast Result of JBC sequence

SX#	Inducible data			Possible genes based on homology (BLASTX)
	Blast	BTH	JA	
2A2		−	+	No homology
2A3	−	+	++	Putative Beta-ketoacyl-CoA synthase
2A4	−	+	++	Low homology (9E-5) with an unknown protein from Arabidopsis
2A8	+	+	++	No homology
2A10	−	+	+	No homology
2A12	−	+/−	++	gb|AAF21081.1|AC013258_19 (AC013258) unknown protein [Arabidopsis thaliana]
2B1	−	+/−	+	No homology
2B7	−	+/−	+	1. hypothetical protein from Arabidopsis (5E-38) 2. cytokinin oxidase-like protein (Arabidopsis) (7E-24)
2B8	−	+/−	+	hypothetical protein from Arabidopsis (5E-18)
2B9	−	−	++	No homology
2C1	+	−	−	No homology
2C3	++	+	−	RUBISCO activase
2C4	+	−	+	=2F8
2C12	++	+	+	MAP kinase (high homology one from maize)
2D2	+ +	++	−	(AC016661) Putative ankyrin (arabidopsis)
2D7	+	−	+	(S39045) Zinic-finger protein from wheat (WZF1) Minwoo
2D10	+/−	−	+	Hypothetical protein from Arabidopsis (4E-6), 24/32 (75%)
2E2		+/−	+	(Z99707) MAP3K-like protein kinase from Arabidopsis
2E3	−	−	+/−	Not sequenced
2E4	+++	−	++	No homology
2E7	R	++++	+++	Low homology: hypothetical protein from Arabidopsis
2E8	−	−	+	No homology
2E11	−	+	++	NAD-malate dehydrogenase
2F6	++	+/−	+	Oryza sativa mRNA for osNAC6 protein (E-155)
2F7	−	+	++	No homology
2F8	+++	−	++	Beta-ketoacyl-CoA synthase
2F10	R	−	+	1. An unknown protein from Arabidopsis 2. Ca+2-binding EF hand protein from soybean 3. ABA induced protein from rice
2F11	+	+	++	= 2A12
2G1	++	+/−	++	No homology
2G5	R	−	−	Chlorophyll A/B binding protein
2G6	++	−	++	(AF225703) RSH2: Arabidopsis Rel/SpoT homology

Add SX3A4

SX2B7 - 2D8

SX1F1

For delete redundancy

Aug 5. MAP Kinase ($2C_2$) Screening again.

Some (ca 10) weaks signal dots → Continue

Aug 15. Northern

ABJS 1
HW 1
blast 7 #1
Southern 2 #
} L34sp (specific probe obtained by PCR)

ABJS 2
HW 2
blast 9 #
Southern 4
} L68sp (specific probe)

phosphoImager's scan: nothing bands remained.

→ ? Washing problem.

→ ? Blots problem. (too old blots)

Aug 21

★ Library screening with L80 (partial or cDNA insert, 1 kb or so)
probe ~~DNA~~ was checked by gel. ★

★ Northern

ABJS1
HW1
Blast 6#
Southern 2
} L34 sp

ABJS2
HW2
Blast 10 (1st time use)
Southern 1 (1st time use)
} L68sp

9/30
↓
10/2

Successfully excised all phagemid ~~into~~ plasmid.
XL0LR cell + New tube grown in 4B !

Absolutely fresh cells be used.

$M_{11}-1 \doteq [M_{11}-2 = M_{11}-3 = M_{11}-5 = \underline{\underline{M_1}}] = 1.4$ kb

$M_2 \stackrel{?}{\doteq} M_8 = 1.6$ kb

M_3 2.2 kb

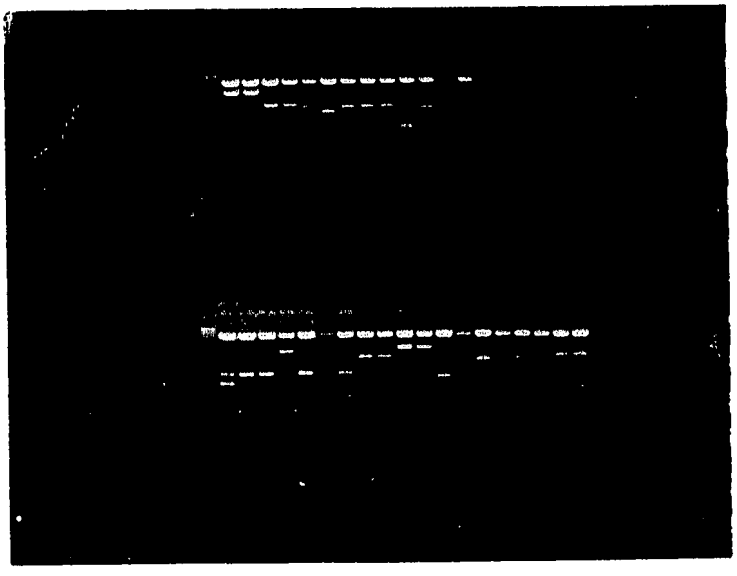
M_4 0.8 kb

$M_5 \stackrel{?}{\doteq} M_6 = 1.3$ kb.

R_1 ~~3~~ ~~1~~ ~~kb~~
1.4
R_2 ~~2.4~~ kb

$R41-1 \neq -2 \neq [\underbrace{-3 = -4 = -5}_{\text{may be true}}]$ → 1.5 kb

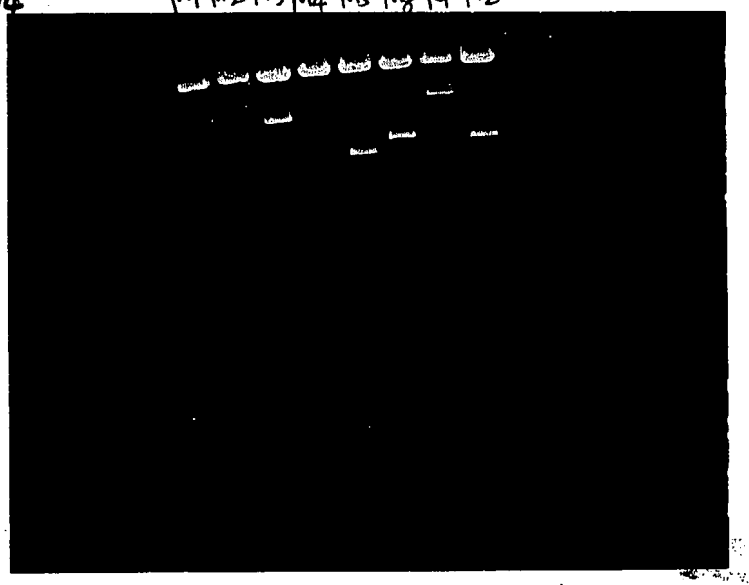
$R51-1 \neq -2 \neq -3 = -4$



10/4

Min: prep.	Final Conc.	
M_1	0.16 ug/ul	1.85
M_2	0.4	2.0
M_3	0.36	1.62
M_4	0.24	1.84
M_5	0.25	1.7
M_8	0.22	1.8
R_1	0.11	0.2
R_2	0.20	1.8

10/4 M_1 M_2 M_3 M_4 M_5 M_8 R_1 R_2



10/5 Send 6 samples for sequence <To Little Rock>

No. 6 M_2 = M_8 $\overset{?}{=}$ 2 C_{12} (need further sequence or digestion)

No. 1 M_3 — wrong! → 18S RNA

2 M_5 partial ? = M_2 or M_8

3 M_8 = M_2

4 R_1 fuller length $\overset{?}{=}$ L80

1/10 ✱ Rnase gel / RNA blotting // for Logic's (CC) Test

CCBT1 - CCBT3

$E_0\ E_1\ E_3\ E_{24}\ C_1\ C_2\ C_3\ C_4\ C_5\ C_6\ B_3\ B_0\ B_0\ B_0\ AV_3\ AV_1\ AV_2\ AV_3\ AV_4\ V_0\ V_1\ V_2\ V_3\ V_4$

9:30 AM

Generating Gene-specific probe (for Northern) or Conserved probe / screening homology

5' MAP 5'- or 3'- specific (Name) Conserved

L44L: $E_{co}RI$ — KpnI — Kpn — ✓ L44L (Kpn) — How long ?

R (L80): 5' ① ② ③ — PstI $E_{co}RI$ Pst Pst Pst — R_1(Pst)①: E+pst, small fragment; ✓ R_1(Pst)②

M2: SacI — SacI — 430 ⇒ M2(SacI): 430

(note 2C12 covers domain X, XI, so it is not gene-specific)

L68: PstI — L68sp — pst+T7 (620+) — T7 — ? ✓ L68sp+T7: product doesn't work well ⇒ L68(pst): 620

L34: L34sp — T7 — ✓ PCR: L34sp+T7 (works!)

Weekend ① Trial Proposal for NOVATIS's Corporation

Jan 10

(1) Northern blotting

ABA – BTH – JA

(3×7: 0, ½, 1, 2, 4, 6, 12 hr)] 2 sets

SA – Wounding – Avir – V.r

7 7 5 (0, 1, 2, 3, 4 days)

Blot#s name : All-in-One 1#, 2#

2 tubes { 1# 150 ng/μl × 400 μl
2# 600 ng/μl × 500 μl

(2) Southern blotting New ~~DNA~~ total r.n.a from Drew using CTAB met

3μg digested by EcoRI, HindIII

(perfect digestion)

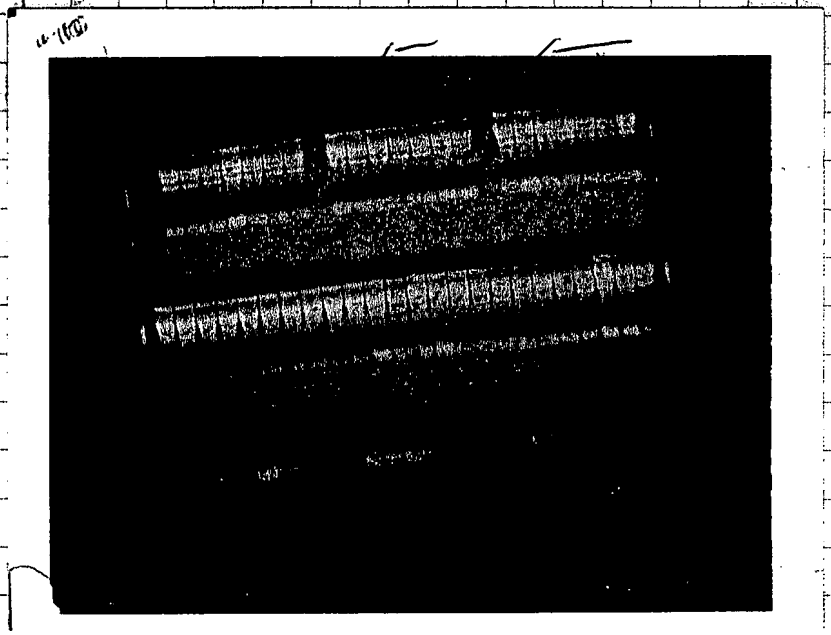
SEH-5, -6, -7, -8

Repeat

(3) Fusion construction → ligation → ~~repeat~~ repeat from ligation → transformation

(see Jan 3 for detail)

(1) ; Picture attached ;



Department Plant Pathology

Subject Rice Defense gene Characterization

Name Lizhong Xiong

Address Rose APC 215

2001.3 —

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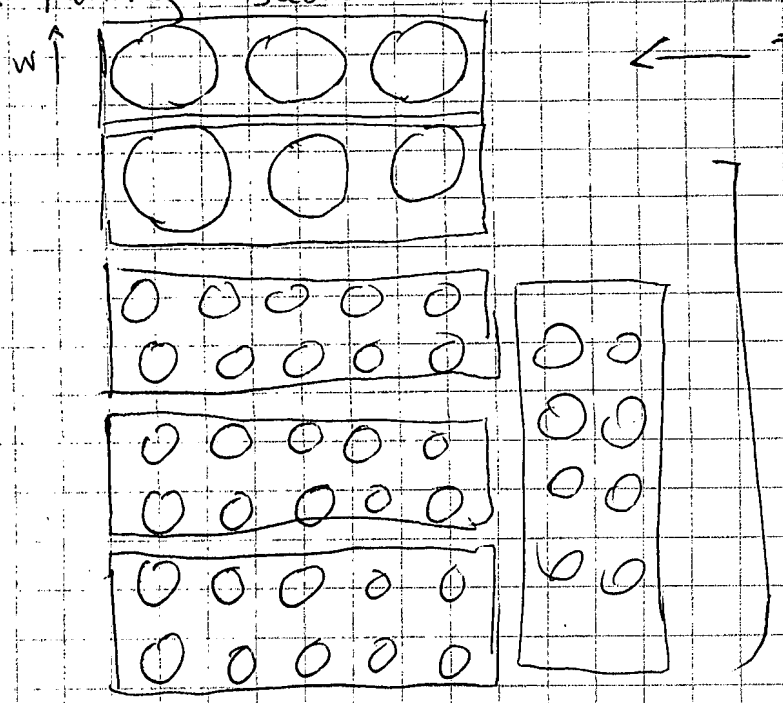
5.18. Sumary of Transformation efficiency

Construct	Resistant calli	Total	shoots ~~of~~ obtained	Total
F2-N	18/37	84/276	1	
	12/41			
G2-N	24/29	119/301		
	22/32			
	17/26			
H2-N	26/41	107/244		
	28/42			
H2-D	3/46	7/263		
	1/42			
C3-D	6/52	9/312		
	2/47			
C3-N (?)	2/36	7/282		
	0/-			
	4/51			
G2-HJ	1/42	9/317		
	2/40			
	2/38			
	0/-			
	0/-			

5/12

* Planting Seeds



purpose : Stress Test for M_2 * large pots are control for transgenic line

1* Salt 150 - 200 mM NaCl

Root & leaf 0. ~~0.5~~ 1. 3. 6. 12. 24. 72hr. 7d.

2* Cold (25° → 4°c :

0 3h 6h 12h 24h

or 28° → 4° for 24h → 28°.

0' 3h' 6'h 12'h 24h

3* Drought

Stop water supply (wet soil)

0 day 1d 2. 3 d 4d ← water content

4* Senescence (chlorophyll content?)

* sampling : small scale in 1.5 ml tube (RNA)

medium scale in 15ml tube (protein)

Order Nylon membrane !

D. TRIAL Western / plant protein
→ ABA-induced. Wounding induced. Blast fungus induced.
→ Extracted w/ Lab protocol for tobaco

E. TRANSGENIC "F_2" (M_2 - DsRNAi)
2 stages Experiment
STAGE I: Copy No. (Southern) x 1 enzyme and / screening all 40 lines
Expression of DsRNAi | 1st Hybridized w/ sequ. on DsRNA
| 2nd - - - - w/ sequ. NOT on DsRNA.

STAGE II: Matured plants (w/ 1 copy and expressed DsRNA)
✡ leaf segment → blast fungas (Dot inoculation)
(Note: ~~not~~ 18/1, ask Min for fungas)
✡ Intact leaf on plant → spay ABA.
other treatment using leaf segment if possible
✡ phenotype Recording for all lines (all constructs).

Only lines showing that endogenous M_2 is inhibited to be induced carry on to T_1 generation.

F. TRANSGENIC "H_2"-N / H_2-D
STAGE I: Same as "F_2"
STAGE II: Same as "F_2" (Focus on blast fungas)
Expected lines: Inhanced Resistance. ✡

G. TRANSGENIC Line "G2" - L4L DsRNAi
STAGE I: Same as in E except:
Sampling for RNA at both 8AM / 9PM
endogenous speci

6.7

1. TRANSfer seedlings (H2 #) (C3-N#

2. Sampling: Cold-RC -24hr.
   Salt 48h. (leaf & root) | AM
   Drought pm3:00 (2day)
   plus
   F2-1 — 22

3. Extract RNA for all Samples, Conc. not determined.

4. PCR for M2-deletion/splicing

   Drew1, Drew2 plamid M1, plasmid M12 H2O

   primer: RTM2F RTM12R (product length of M2 should be 1.0 kb)

   Taq: Home made (0.5 ul) in 50ul Vol
   added after temp. reach 95°C

6.8

1. CE PCR

2. TRANSfer F16-HJ (only one) Resistant callus to Regeneration Medium.

3. Salt 3d.
   drought 3d. | Sample

4. prepare talk in Mon. (SERK)